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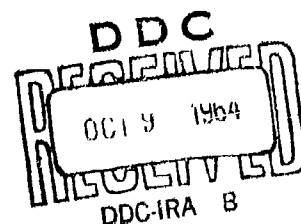
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TECHNICAL MANUSCRIPT 160

IN VIVO GROWTH CURVES  
OF BACILLUS ANTHRACIS

SEPTEMBER 1964



UNITED STATES ARMY  
BIOLOGICAL LABORATORIES  
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U.S. ARMY BIOLOGICAL LABORATORIES  
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TECHNICAL MANUSCRIPT 160

IN VIVO GROWTH CURVES OF BACILLUS ANTHRACIS

W.I. Jones, Jr.  
B.U. Ross  
F. Klein  
J.S. Walker  
R.E. Lincoln  
B.G. Mahlandt  
B.W. Haines

Process Development Division  
DIRECTOR OF DEVELOPMENT

Project 1C522301A082

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### ABSTRACT

Previous investigators have noted that the number of bacteria in the blood of animals infected with fatal anthrax is dependent upon the host species and its degree of immunity or resistance. Quantitative measurements of the concentration and distribution of organisms in various tissues and organs of immunized and nonimmunized guinea pigs and rats infected with Bacillus anthracis have been made. These studies have shown that death does not necessarily occur at the peak bacterial concentration in the body.

In either immune or naturally resistant hosts the concentration of organisms in all tissues of the body is lowered. In immunized hosts, bacilli tend to remain localized in the muscular tissues until shortly before death, when a terminal septicemia occurs. There is an apparent inverse time-bacterial concentration relationship present, because the animals with the lowest concentration of organisms in the body live the longest.

During the pre-septicemic phase of the disease, a relatively high concentration of organisms per gram of tissue can be found in the liver as compared to the other organs. Standard biopsy techniques, rather than establishing the presence of the bacilli in the blood, may possibly be used to detect anthrax in its early stages.

# IN VIVO GROWTH CURVES OF BACILLUS ANTHRACIS

Generalized anthrax is typified by an extremely rapid but orderly progress of disease with symptoms and septicemia occurring some few hours prior to death. Klein, et al<sup>1</sup> reviewed earlier literature and presented quantitative data regarding the in vivo growth rate and bacilli level in the blood of immunized and normal guinea pigs during the septicemic stage of anthrax and noted the constancy of these observations regardless of certain treatment variations of the initial challenge dose.

A disease, whose progress is so rapid and terminates so abruptly and drastically, may merit drastic early diagnostic measures. In this study we determined the concentration and distribution of organisms in various tissues and in the whole carcass during the entire course of the disease. From these data we could determine the effect of certain immunization protocols on bacterial level and use them as a possible guide in the use of biopsy to detect anthrax prior to its normal fatal septicemia.

The susceptible 250 to 350 gram Hartley strain guinea pig from the Fort Detrick animal farm, and the highly resistant 200 to 250 gram Norvegicus black rat from Long-Evans stock obtained from the National Institutes of Health animal farm were used.\* One group of guinea pigs was immunized with the Belton-Strange<sup>2</sup> protective antigen prepared by the method of Thorne and Belton<sup>3</sup> and diluted 1:10. The protective antigen was administered by intraperitoneal injection of 0.1 ml on Days 1, 3, 5, 8, and 11. These animals will be referred to as PA-5 animals. In addition, a group of rats, after receiving the initial PA-5 protocol were given a 1.0 ml booster of live vaccine ( $1 \times 10^8$  spores/ml of the lowly virulent 3OR strain of B. anthracis) and will be referred to as PA-5 + LV animals. One week after the completion of their respective protocols the immunized animals were challenged with  $1 \times 10^7$  B. anthracis spores of the highly virulent Vollum strain (Vlp), which were enhanced by treating with egg yolk as described by Kaga.<sup>4</sup> All challenges were by the subcutaneous route. Mean time to death values for all treatments were determined using ten animals per treatment.

Independent data were obtained on each animal and organ. Each six hours after challenge, several animals were serially sacrificed with carbon dioxide (dry ice), skinned and weighed. A blood sample for assay was drawn from the heart, then the spleen, kidneys, liver, lungs and intestinal tract were removed. The remaining carcass of the animal then was weighed and ground in a Hamilton Beach heavy-duty meat grinder and homogenized in the Servall Omni-mixer. Each weighed organ was homogenized

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\* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.

in the presence of suitable diluent by means of the Tri-R stirring apparatus. The samples were assayed immediately. Serial dilutions of each sample were made in gel-phosphate diluent (two per cent gelatin, four per cent  $\text{Na}_2\text{HPO}_4$ , pH adjusted to 6.8 to 7.0) and 0.1-ml or 0.3-ml samples of inoculum were spread on tryptose agar plates containing 0.005 per cent potassium tellurite, which suppresses contamination but not the growth of B. anthracis.

Pooled data collected revealed that nonimmunized pigs, when challenged with  $1 \times 10^7$  B. anthracis spores fortified with egg yolk, exhibit a 1.5-log decrease in organism concentration during the first 12 hours of disease followed by a rapid build-up in all tissues or organs over the next 18 hours until death ensued at 30 hours (Figure 1). However, if the guinea pig had been immunized by the PA-5 protocol, the build-up of organism concentration in all organs was very gradual for the first 36 to 40 hours of the disease, followed by a slight decrease in concentration over the next 6 to 12 hours and terminating in death at 48 hours (Figure 2).

Examination of Figures 1 and 2 and their accompanying tables reveals that protective antigen extends the time to death in the guinea pig some 18 hours and lowers the maximum concentration of organisms in the body approximately one log. It should be noted that anthrax in the nonimmunized guinea pig is accompanied by a marked build-up of organisms in the blood, whereas in the PA-5 immunized hosts, the majority of organisms remain concentrated in the muscular tissue until the last 20 hours of the disease, at which time the organisms tend to become more evenly distributed throughout all tissues and organs. The percentage distribution and organism concentration tables show that organisms are more likely to be isolated in the liver in the early stages of the disease and in the blood during the latter half of the course of the disease. This study substantiates earlier findings that immunized guinea pigs dying from anthrax had a lower bacterial concentration in their blood terminally than did nonimmunized guinea pigs. Examination of the data shows further that once the disease is established, the number of organisms per unit of body weight is always higher in the non-immunized guinea pig.

Since Klein, et al., in 1961<sup>1</sup> showed that NIH black rats could not be significantly immunized by the PA-5 protocol, it appeared feasible to compare PA-5 rats with the PA-5 + IV group in the same manner as we did in immunized and nonimmunized guinea pigs. Challenge of the NIH black rat subjected to the PA-5 protocol produces a steady build-up in bacterial concentration during the first 24 hours of the disease, followed by an 18-hour plateau, and resulting in death at 42 hours (Figure 3). If the PA-5 rat was given a live vaccine booster prior to challenge, there was a steady build-up in all organs during the first 24 to 30 hours, followed by a gradual decrease in bacterial concentration in the next 18 hours and culminating in death at 48 hours (Figure 4). Although the greatest percentage of bacteria is localized in the muscular tissues until shortly before death, there apparently is a sufficient number of organisms concentrated in the liver to warrant liver biopsy during the first half of the course of the disease. Diagnosis after the onset of septicemia would naturally be based on blood culture.

# CHALLENGE: $1 \times 10^7$ SPORES $\bar{C}$ EGG SUBCUTANEOUS ROUTE

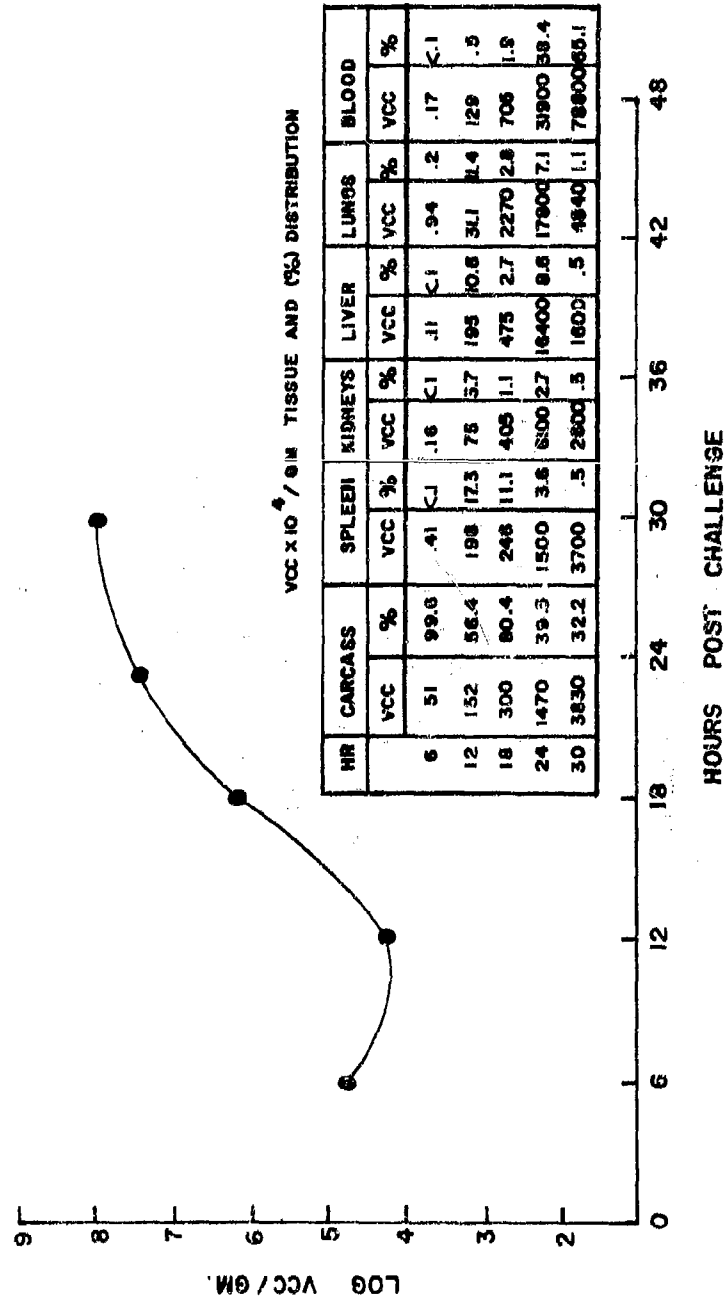


Figure 1. Growth of B. anthracis in Body of Nonimmunized Guinea Pig.

# CHALLENGE: $1 \times 10^7$ SPORES $\bar{C}$ EGG SUBCUTANEOUS ROUTE

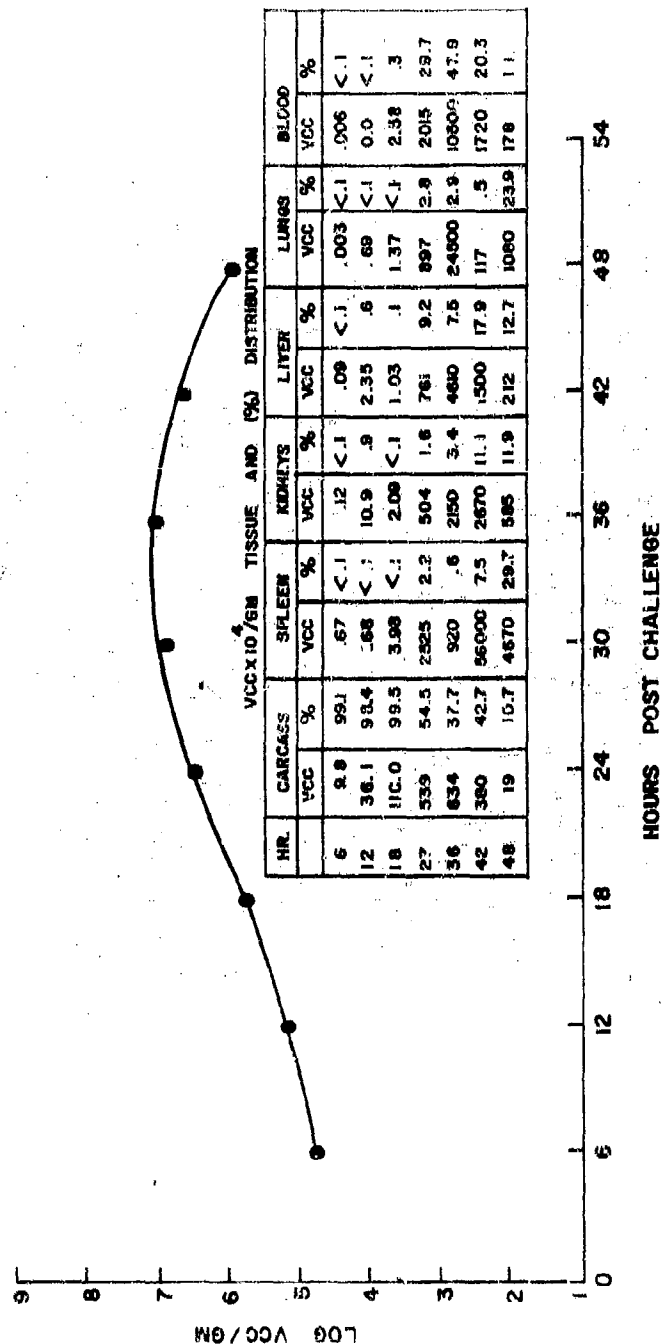


Figure 2. Growth of *B. anthracis* in Body of PA-5 Immunized Guinea Pig.

CHALLENGE:  $1 \times 10^7$  SPORES  $\bar{C}$  EGG SUBCUTANEOUS ROUTE

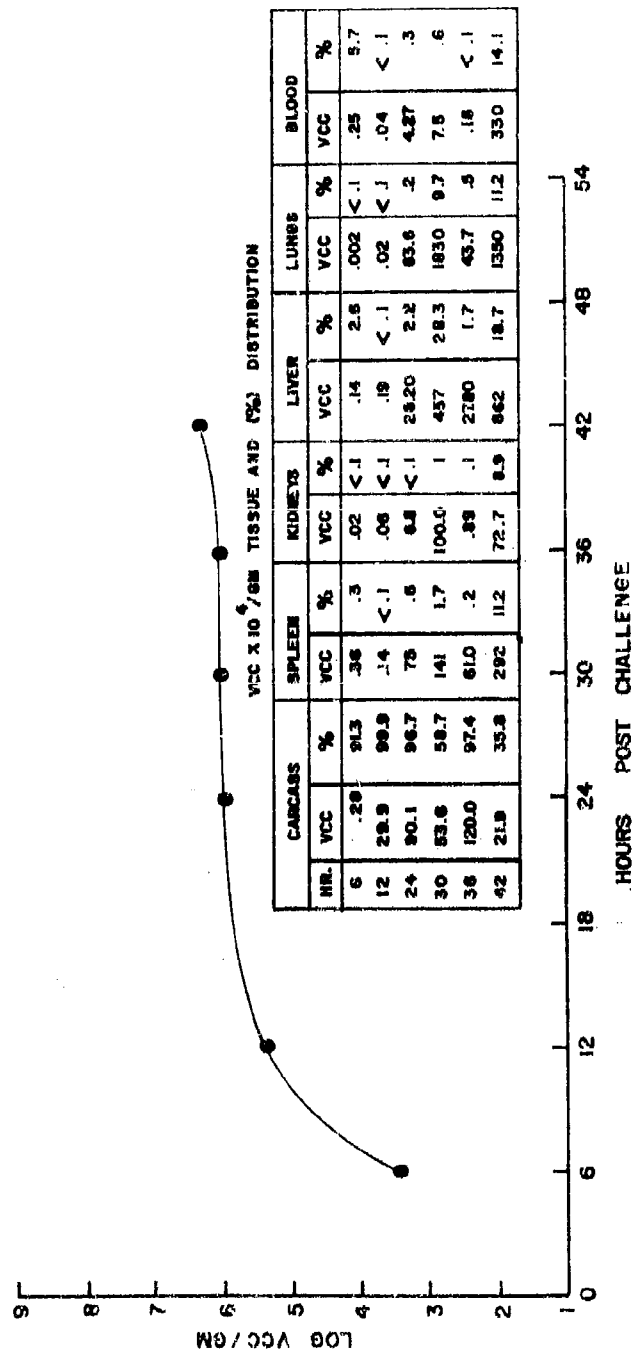


Figure 3. Growth of *B. anthracis* in Body of PA-5 Immunized NIH Rat.

CHALLENGE:  $1 \times 10^7$  SPORES C EGG SUBCUTANEOUS ROUTE

HR.	VCC $\times 10^4$ /GM TISSUE AND % DISTRIBUTION											
	CARCASS		SPLEEN		KIDNEYS		LIVER		LUNGS		BLOOD	
	VCC	%	VCC	%	VCC	%	VCC	%	VCC	%	VCC	%
6	1.13	57.6	527	1.6	.11	<.1	9.60	40.4	3.93	.3	.01	<.1
12	7.5	94.7	2.17	.3	.06	<.1	5.52	4.9	.02	<.1	.08	<.1
24	.89	95.5	1.27	.6	.11	.2	.32	3.3	.09	.1	.001	<.1
30	.76	99.8	.50	<.1	.18	<.1	1.08	<.1	1.00	<.1	1.88	.2
36	14.4	98.7	3.71	.4	.08	<.1	.45	.5	.53	.1	.06	<.1
42	.002	.5	6.21	16.4	.14	.7	.58	13.9	.08	.6	.03	65.9
48	.22	27.9	58.1	61	.06	.2	.98	10.4	.06	.1	.03	.3

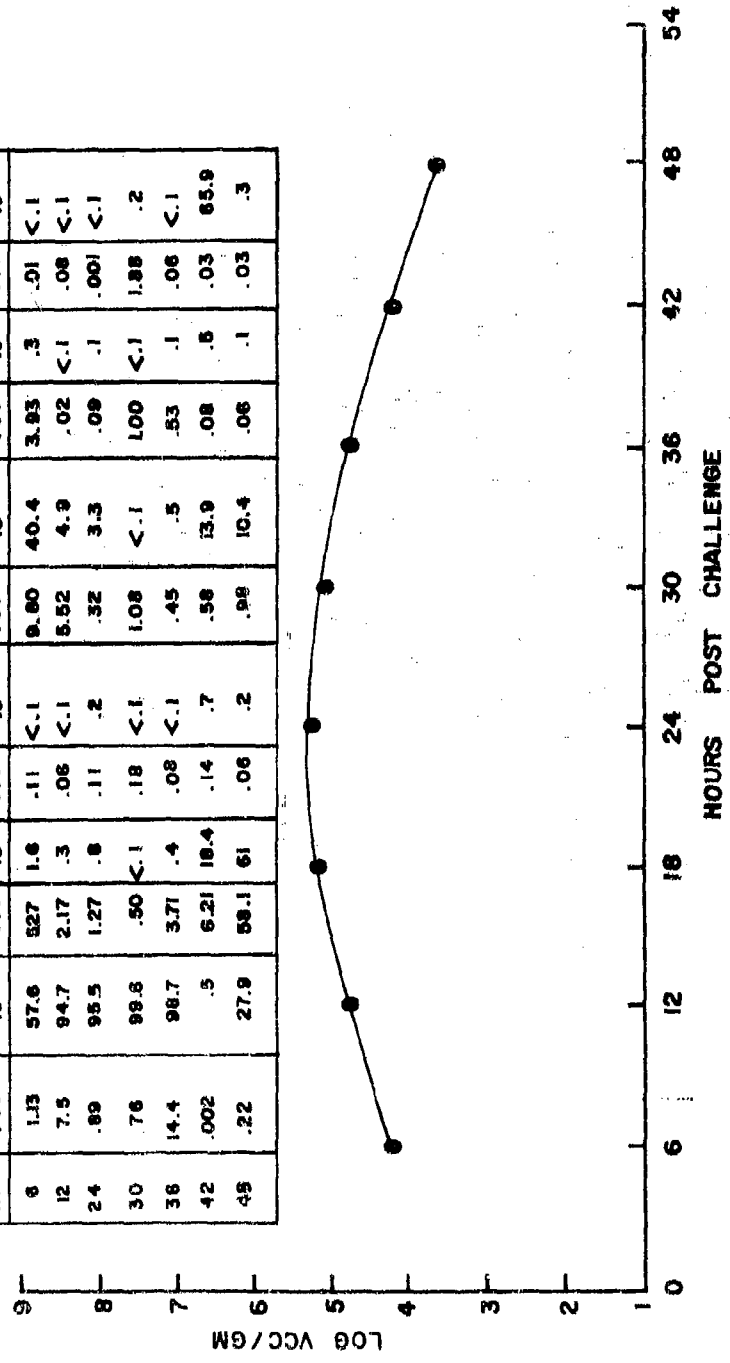


Figure 4. Growth of B. anthracis in Body of PA-5 + LV Immunized NIH Rat.

To determine the effect of the amount of residual blood with its organisms on the number of bacilli isolated from the various organs, an indirect measurement of the blood remaining and assayed in the organs was made as follows: slurries of the various organs and tissues were centrifuged and the amount of hemoglobin in the supernatant fluid determined by the cyanmethemoglobin methods. The hemoglobin values obtained were compared with the amount of hemoglobin found in a given unit of the animal blood. The ratio thus obtained was corrected to a volume of residual blood in the organ. Values obtained showed that the numbers of organisms isolated in the various organs and tissues are not significantly influenced by the residual volume of blood in the excised organs and tissues.

We earlier noted an inverse relationship between time to death and number of bacilli per gram of tissue. One might reasonably expect that more organisms would be required to kill the immunized than the non-immunized host. For one group of 28 guinea pigs, the relationship between time to death, number of organisms per ml of blood, and units of toxin present in the blood are plotted in Figure 5. It is evident that, as the resistance of the host increases as evidenced by increased time to death, both the number of organisms per ml and the units of toxins present in the terminal blood decrease. However, this latter relationship is not a simple arithmetic function. We are still unable to explain why hosts made resistant by immunity die with fewer organisms present or with less toxins present.

Although a septicemia was observed in all test animals, it was pronounced in the more susceptible group of a species. All organs showed a bacterial build-up during some phase of the disease and the rates of build-up in the various organs differed. In general, the organisms tend to remain localized in the muscular tissues of immunized hosts until shortly before death, at which time the organisms become more evenly distributed throughout all tissues and organs. Animals that live the longest generally have the lowest bacterial concentration in the body. Death does not necessarily occur simultaneously with the peak bacterial concentration.

These studies show that not only is it harder to establish anthrax in animals with natural or induced immunity, but once established, it is more difficult to detect anthrax since the number of bacilli in the immunized host is reduced and the septicemia is both delayed and terminates earlier. We suggest that biopsy of the liver appears to be the most feasible method of diagnosing anthrax early in the course of disease, while the late septicemic stage will be obvious in the blood.

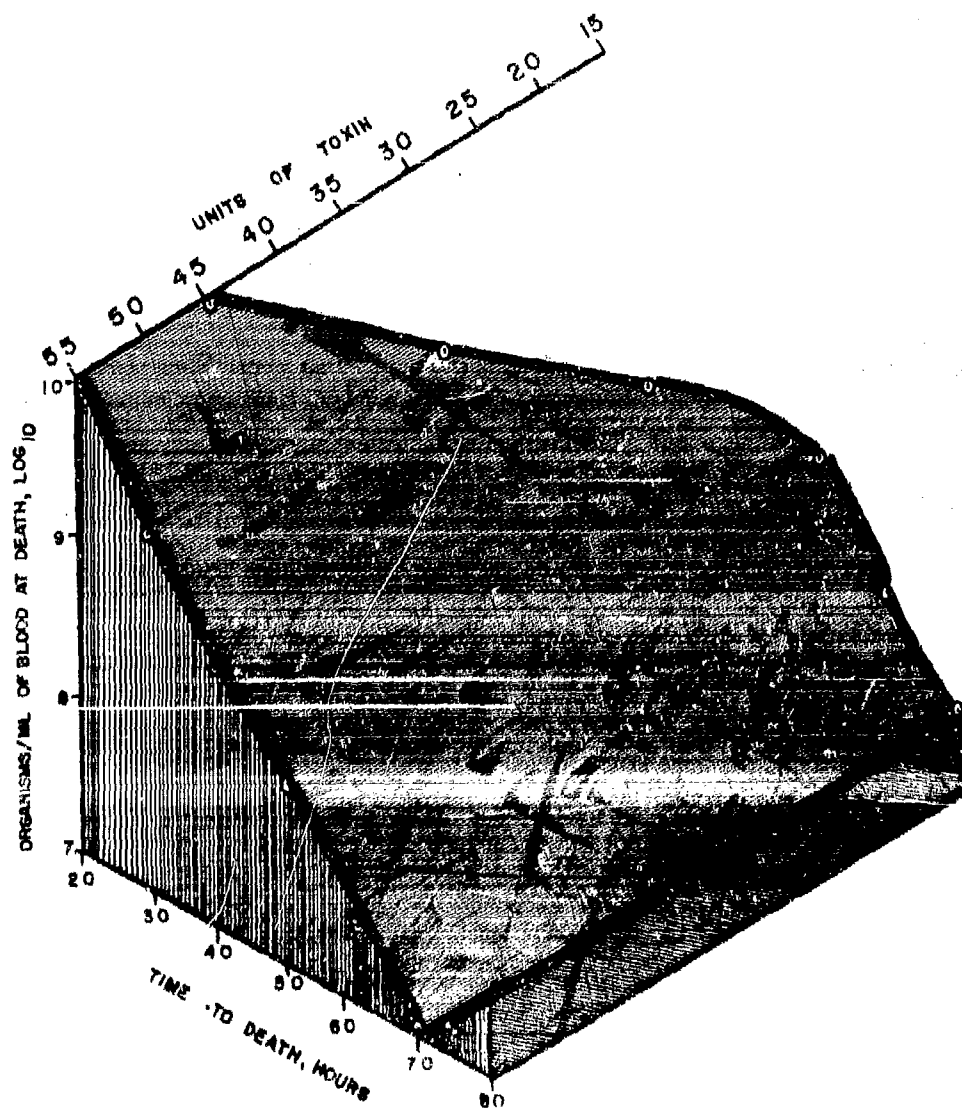


Figure 5. Relationship Among Three Variables, (1) Time to Death, Hours, (2) Organisms per Milliliter of Blood at Death, and (3) Units of Toxin in Terminal Blood in Guinea Pigs Dying of Anthrax. Data plotted are taken from the regression of (2) on (1) and (3) on (1).

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